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## Retrovirus vector-mediated transfer of functional HIV-1 regulatory genes.

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Replication of the human immunodeficiency virus depends on the expression of its regulatory genes. We have constructed three plasmids, based on the retrovirus vector LXS<sub>N</sub>, that contain the tat, rev, and env (pLTRES<sub>N</sub>), the rev and env (pLRES<sub>N</sub>), and the nef (pLnef<sub>N</sub>) genes of HIV-1. In a two-step virus rescue protocol, during which introns are removed from the DNA fragments inserted into pLXS<sub>N</sub>, these plasmids were used to establish amphotropic retrovirus vector producer lines for the transfer of tat (Ltat<sub>N</sub>), rev (Lrev<sub>N</sub>), and nef (Lnef<sub>N</sub>). These vectors have titers greater or equal to 10<sup>6</sup> CFU/ml and efficiently transduced each of these genes into a variety of human and murine cell lines. Representative populations of cells constitutively expressing the tat and rev genes were obtained. Cell lines transduced with Ltat<sub>N</sub> were able to trans-activate an HIV-LTRCAT construct, indicating the presence of a functional Tat protein. Similarly, cells transduced with Lrev<sub>N</sub> were able to rescue a rev- HIV-1 provirus, indicating the presence of a functional Rev. We also used Lnef<sub>N</sub> to obtain clones of cells expressing Nef. Our results indicate that these retrovirus vectors are useful reagents for the efficient transfer of functional Tat, Rev, and Nef and for the establishment of cell lines constitutively expressing these genes.

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## Retrovirus Vector-Mediated Transfer of Functional HIV-1 Regulatory Genes

J. VICTOR GARCIA<sup>1</sup> and A. DUSTY MILLER<sup>2</sup>

### ABSTRACT

Replication of the human immunodeficiency virus depends on the expression of its regulatory genes. We have constructed three plasmids, based on the retrovirus vector LXS<sub>N</sub>, that contain the *tat*, *rev*, and *env* (pLTRES<sub>N</sub>), the *rev* and *env* (pLRES<sub>N</sub>), and the *nef* (pLnef<sub>N</sub>) genes of HIV-1. In a two-step virus rescue protocol, during which introns are removed from the DNA fragments inserted into pLXS<sub>N</sub>, these plasmids were used to establish amphotropic retrovirus vector producer lines for the transfer of *tat* (Ltat<sub>N</sub>), *rev* (Lrev<sub>N</sub>), and *nef* (Lnef<sub>N</sub>). These vectors have titers greater or equal to 10<sup>6</sup> CFU/ml and efficiently transduced each of these genes into a variety of human and murine cell lines. Representative populations of cells constitutively expressing the *tat* and *rev* genes were obtained. Cell lines transduced with Ltat<sub>N</sub> were able to trans-activate an HIV-LTRCAT construct, indicating the presence of a functional Tat protein. Similarly, cells transduced with Lrev<sub>N</sub> were able to rescue a *rev*<sup>-</sup> HIV-1 provirus, indicating the presence of a functional Rev. We also used Lnef<sub>N</sub> to obtain clones of cells expressing Nef. Our results indicate that these retrovirus vectors are useful reagents for the efficient transfer of functional Tat, Rev, and Nef and for the establishment of cell lines constitutively expressing these genes.

### INTRODUCTION

**H**UMAN IMMUNODEFICIENCY VIRUS (HIV) is a human retrovirus that causes acquired immune deficiency syndrome (AIDS).<sup>1</sup> Unlike murine retroviruses, HIV encodes a series of nonstructural proteins that regulate virus replication.<sup>2</sup> The nonstructural genes of HIV have been divided into two groups: regulatory and auxiliary genes.<sup>3</sup> The regulatory genes of HIV are *tat*, *rev*, and *nef*. Tat and Rev are necessary for viral replication and act on HIV via sequence-specific elements present in the viral RNA.<sup>4-9</sup> Tat increases the overall levels of all viral mRNAs and Rev promotes the transport of unspliced and singly spliced viral mRNAs from the nucleus to the cytoplasm.<sup>10-16</sup> Unlike Tat and Rev, Nef is not essential for virus propagation *in vitro* and its function in virus replication is controversial.<sup>17-19</sup> Nef was reported to downregulate transcription via the viral long terminal repeat (LTR).<sup>18,20,21</sup> However, other investigators have reported that Nef has no influence on viral LTR transcription.<sup>22,23</sup> Most of the studies on these genes

reported to date have used transfection as the means to express these genes *in vitro*. Although important information has been obtained by this technology, it has two significant disadvantages: (1) in most cases, multiple copies of plasmid are introduced at the same time into each cell, resulting in artificially high levels of the protein; and (2) most of the cell types used in AIDS research (e.g., lymphocytes) are difficult to transfect. Retroviral vectors serve as an efficient means to stably introduce foreign genes into a variety of target cells from multiple tissues both *in vivo* and *in vitro*.<sup>24-26</sup> To facilitate the study of these regulatory genes we have established and characterized retroviral producer lines for each of these genes. Here we describe the construction of these retrovirus producer lines and show that they can efficiently transduce these genes into a variety of cell types. Cells transduced by these retrovirus vectors express functional Tat, Rev, and Nef proteins. Our results show that these vectors promote efficient gene transfer and are useful to establish either individual cell clones or representative populations of cells constitutively expressing the regulatory genes of HIV.

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## MATERIALS AND METHODS

### Cells and culture conditions

Monolayer cell lines (NIH 3T3 TK<sup>-</sup>, HeLa, PA317,<sup>27</sup> and PE501<sup>28</sup>) were grown in Dulbecco's modified Eagle medium (DMEM) with high glucose (4.5 g/liter) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), penicillin, and streptomycin at 37°C in a 10% CO<sub>2</sub> incubator.<sup>27</sup> Jurkat (obtained from D. Geraghty, Fred Hutchinson Cancer Research Center, Seattle WA), HPBALL (obtained from E. Clark, University of Washington, Seattle, WA), Molt4, 8166 (obtained from M. Galatin, ICOS, Bothell, WA) SupT1, AA2, U937 (donated by J. Hoxie, M. Herschfield, and H. Koren and obtained from the NIH/AIDS Research and Reference Program), and CEM (ATCC) human cell lines were grown in RPMI-1640 medium supplemented with 12.5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 10% fetal bovine serum, penicillin, streptomycin, and amphotericin B (Fungizone) at 37°C in a 5% CO<sub>2</sub> incubator.

### Retrovirus vector construction

All HIV fragments were cloned into the retroviral vector pLXSN,<sup>28</sup> using standard techniques as follows: pLTRESN was constructed by ligating an *EcoRI*-*XhoI* fragment (nucleotides 5750–8914) from the HIV-1 isolate SF2 (obtained from P. Luciw, University of California, Davis)<sup>29</sup> into the *EcoRI* and *XhoI* restriction sites of pLXSN. This 3164-bp fragment contains the *tat*, *rev*, and *env* genes of HIV. pLTRESN was constructed by removing 194 bp of the first *tat* exon from pLTRESN by digestion with *EcoRI* and *MluI* and religation. pLnefSN has been described<sup>30</sup> and was constructed by ligation of a *BglII* fragment (nucleotides 8504–9574) from HIV-1 SF2 into the *BamHI* restriction site in pLXSN.

### Generation of stable vector-producing cell lines

Stable retrovirus vector-producing cell lines were generated by calcium phosphate transfection, essentially as previously described.<sup>28</sup> Briefly, PE501 ecotropic packaging cells were transfected with 10 µg of plasmid DNA. Ecotropic virus harvested from the PE501 cells was used to infect PA317 cells. Individual cell clones (5–15) selected in medium containing G418 (1.5 mg/ml, 50% active; GIBCO, Grand Island, NY) were isolated from plates with a small number of colonies, using glass cloning rings. Individually isolated clones were assayed for virus titer and for the structure of the integrated provirus (see below). Virus titer was determined by infection of NIH 3T3 TK<sup>-</sup> cells ( $5 \times 10^5$ ) with 1–5 µl of the filtered PA317 conditioned medium as described above. Infected cells were split (1:10 and 1:100) and cultured in medium containing G418 (1.5 mg/ml). Five to 7 days later cell colonies were stained with Coomassie blue and counted.

### Retroviral gene transfer into human cells

HeLa cells were infected as described above for NIH 3T3 TK<sup>-</sup> cells, except that selection was carried out in G418 (2 mg/ml; 50% active) for 7 to 10 days. CEM, Jurkat, Molt4, 8166, SupT1, HPBALL, U937, and AA2 cells ( $0.5\text{--}1 \times 10^6$ ) were

incubated overnight with the appropriate virus supernatant (0.1 to 2.5 ml) in medium (4 ml) containing Polybrene (4 µg/ml). Virus and Polybrene were removed by centrifugation and cells plated in fresh medium (4 ml) containing G418 (1.5–2 mg/ml; 50% active). Ten to 14 days later cells were transferred to 10-cm dishes and 6 ml of fresh medium with G418 was added. Five to 7 days later cells were split (1:20 or 1:10) into fresh medium containing G418. Cultures were then passage as necessary in medium without G418.

### DNA and RNA analysis

Genomic DNA was prepared from individual PA317 producer clones and analyzed for provirus structure by Southern blotting after digestion with *XbaI*, which cuts once in both LTRs as described.<sup>25,26</sup> RNA was prepared by the guanidium thiocyanate method<sup>31</sup> and analyzed by Northern blotting as described.<sup>26</sup> DNA and RNA blots were probed with a <sup>32</sup>P-labeled *neo*-specific DNA probe.

### Analysis of *Tat*, *Rev*, and *Nef* expression

Expression of functional *Tat* in adherent cells was determined by measuring trans-activation of a transfected HIV-LTR-CAT construct (pH3LTRCAT, provided by I. Chen, University of California, Los Angeles). *Tat* expression in human T and B cell lines was determined by measuring trans-activation of the pH3LTRCAT construct after electroporation as follows: suspension cells ( $1 \times 10^7$ ) were collected by centrifugation and grown in 25 ml of medium in a T75 flask for 2 days. Electroporation and CAT assay conditions were similar to those described by Cann *et al.*,<sup>32</sup> except that 200 V was used. *Rev* expression was monitored by rescue of p24 production by a *rev*<sup>-</sup> HIV-1 provirus (JR-CSF delta-Sal; provided by S. Arrigo and I. Chen, University of California, Los Angeles)<sup>33</sup> after transfection (monolayer cells) or electroporation (T and B cells) as described above. Particle-associated p24 was determined with an antigen trap assay kit (Coulter [Hialeah, FL] or Abbott [Abbott Park, IL]), following manufacturer instructions. *Nef* expression was determined by immunoprecipitation and Western blot analysis after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using a rabbit antiserum against recombinant *Nef* produced in bacteria (obtained from L. Ratner, Washington University, St. Louis, MO). Briefly, cells were lysed in radio-immunoprecipitation (RIPA) buffer containing protease inhibitors and 1 µl of the anti-*Nef* antiserum was added. Samples were incubated on ice for 1 hr and immunocomplexes were separated by binding to protein A-Sepharose (Pharmacia, Piscataway, NJ). Samples were then separated by SDS-PAGE on a 12% gel, transferred to nitrocellulose, and probed with the anti-*Nef* antiserum (1:500 dilution). Blots were developed by using an alkaline phosphatase labeled goat anti-rabbit antibody (Promega, Madison, WI).

## RESULTS

### Characterization of retrovirus producer lines

The strategy followed to make the retroviral producer cell lines involved the transient rescue of virus from an ecotropic

retrovirus packaging cell line, which in turn was used to infect an amphotropic packaging line. This general approach yields stable vector-producing cell lines containing single integrated proviruses. At the same time, introns present in the inserted fragments are removed during the single round of retrovirus replication (Fig. 1). In the case of pLTRESN, six PA317 clones were analyzed. Four had the correct (e.g., spliced) proviral insert, and two had rearranged provirus inserts (data not shown). The resultant LtatSN producer lines had titers ranging from  $4 \times 10^5$  to  $3 \times 10^6$  CFU/ml. Clone 2B4 had the highest titer ( $3 \times 10^6$  CFU/ml) and was used for the experiments described below. To make the Rev retrovirus producer line, a 194-bp fragment (*EcoRI* to *MluI*) of the first exon of *tat* was removed from pLTRESN to generate pLRESN. Following the same strategy described (Fig. 1), 10 independent PA317 clones were analyzed for proviral structure and titer. Nine of the proviruses were approximately 3400 bp in length (Fig. 2A), indicative of a provirus that has deleted the intron containing most of the HIV *env* gene. This was further confirmed at the RNA level by Northern blot analysis (Fig. 2B). The titers of the PA317/LrevSN producer lines ranged between  $10^3$  and  $10^6$  CFU/ml. Clone NA3 had the highest titer ( $1 \times 10^6$  CFU/ml) and was used in all subsequent experiments. The PA317/LnefSN producer line was prepared in the same way and virus titers ranged between  $6 \times 10^4$  and  $1 \times 10^6$ . Clone 11 had a titer of  $10^6$  CFU/ml and was used in all experiments.

# HIV long terminal repeat trans-activation by Tat-expressing cell lines

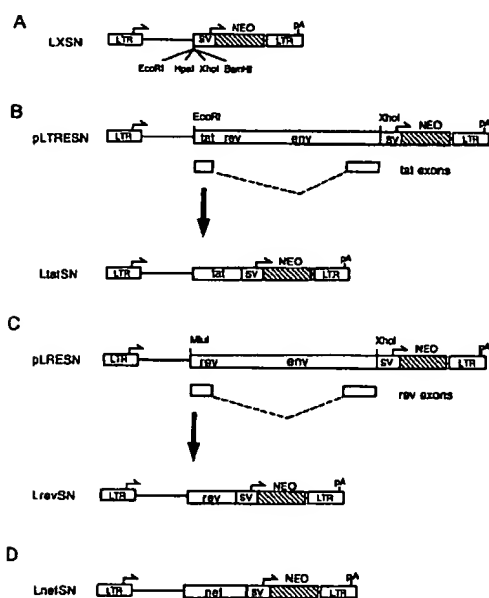
To determine if LtatSN produced virus containing a functional *tat* gene, U937, AA2, HPBALL, SupT1, HeLa, and NIH 3T3 TK<sup>-</sup> cells were transduced with LtatSN. After selection in G418, representative populations of cells were tested for Tat expression using a functional assay based on HIV LTR trans-activation. pH3LTRCAT, a plasmid that contains the *cat* gene under the control of the HIV LTR, was transfected (adherent cell lines) or electroporated (suspension cell lines)<sup>32</sup> into cells transduced with LtatSN or control viruses. These results show that retroviral-mediated gene transfer by itself does not affect the basal level of LTR trans-activation. Only in cells transduced with LtatSN is the LTR efficiently trans-activated. Extracts from all cell lines transduced with LtatSN showed markedly higher CAT activities than corresponding cells transduced with a control vector that expressed only *neo* (Fig. 3A and B) or with the *rev* vector (Fig. 3B).

# HIV *rev*<sup>-</sup> mutant complementation by Rev-expressing cell lines

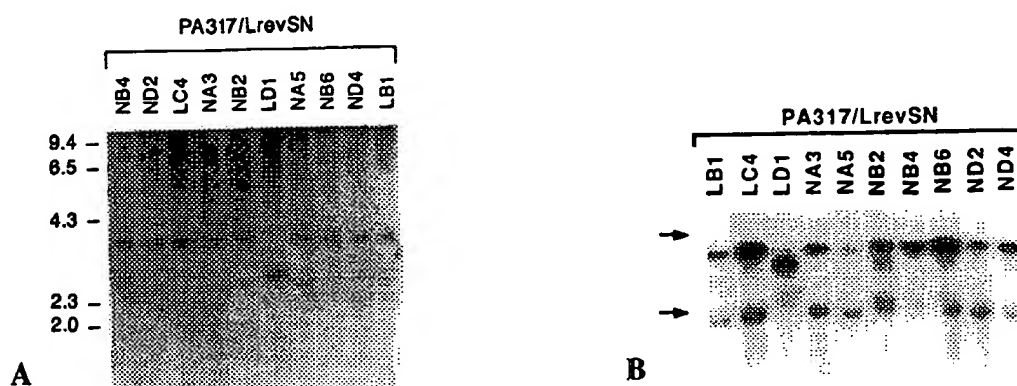
To monitor Rev expression in cells transduced with LrevSN, the *rev*<sup>-</sup> mutant complementation assay described by Arrigo *et al.* was employed.<sup>33</sup> In the absence of Rev, RNA transcripts coding for the *gag*, *pol*, and *env* genes are retained in the nucleus and not translated into proteins.<sup>12,14,33</sup> In this assay a *rev*<sup>-</sup> mutant of JR-CSF (delta-Sal) was introduced into human T, B, and monocytic/macrophage cell lines and HeLa cells transduced with LrevSN or with a control virus. Only cells transduced with LrevSN complemented the *rev*<sup>-</sup> provirus as determined by the accumulation of a significant amount of p24 in the culture supernatant (Table 1). Although most control cell lines produced a low level of p24, the only two control non-T cell lines tested, AA2 and HeLa, had 3–30 times more background p24, suggesting a small level of Rev-independent RNA leakage to the cytoplasm in these cells. These results indicate that LrevSN can transduce a functional *rev* gene able to fully complement a *rev*<sup>-</sup> HIV-1 provirus.

# Analysis of Nef expression after retroviral-mediated gene transfer

Two independent groups have reported difficulty in establishing cell lines constitutively expressing Nef, raising the possibility that Nef expression might be toxic.<sup>17,34</sup> Using retrovirus-mediated gene transfer, we have shown that populations of human cells constitutively expressing functional Nef can be obtained.<sup>30</sup> However, these populations of cells have been established under conditions that favor integration of a single provirus per cell. They also were maintained in culture for 2–3 weeks prior to analysis for Nef expression.<sup>30</sup> To determine the efficiency of *nef* transfer, we transduced NIH 3T3 TK<sup>-</sup> cells with LnefSN. We then isolated individual G418-resistant clones and determined Nef expression levels. Extracts from four of the five clones analyzed showed similar levels of a protein of ~29 kDa that reacted with Nef antiserum (Fig. 4). The fact that four of five individual clones analyzed express Nef indicates that transduction of the *nef* gene is efficient and further suggests that Nef expression per se is not deleterious to these cells.



**FIG. 1.** Retroviral vector construction. (A) pLXSN<sup>28</sup> was the parent vector into which HIV fragments were inserted. In all cases, HIV sequences were driven by the Moloney murine leukemia virus long terminal repeat (LTR). The selectable marker (*neo*) is driven by the simian virus 40 (SV40) promoter. (B) Tat-expressing vector before (top) and after (bottom) introns are removed. (C) Rev-expressing vector before (top) and after (bottom) introns are removed. (D) Nef-expressing vector.



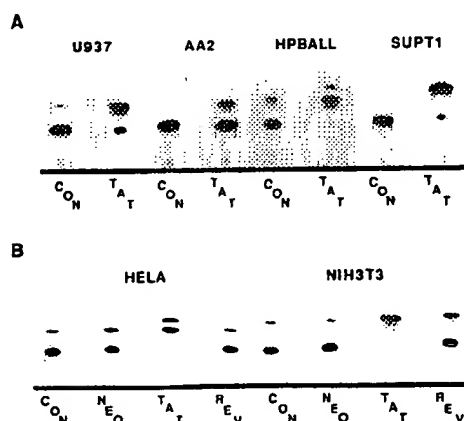
**FIG. 2.** Proviral structure of the LrevSN producer lines. (A) Southern blot analysis of LrevSN amphotropic producer lines. The band that migrates at approximately 3400 bp corresponds to LrevSN. Molecular weight markers are indicated on the left (kbp). (B) Northern blot analysis of LrevSN amphotropic producer lines. The high molecular weight band corresponds to the viral RNA encoding the *rev* gene, the lower molecular weight band corresponds to the SV40-driven *neo* gene. Arrows indicate the position in the gel of the ribosomal RNA (28S and 18S). Both blots were probed with a *neo*-specific DNA probe.

## DISCUSSION

Efficient replication of HIV is dependent on the expression of its regulatory genes.<sup>2,3</sup> To further advance our understanding of how these genes affect both HIV replication and host cell gene expression, a series of plasmids based on the retrovirus vector pLXSN were constructed that contain the *tat*, *rev*, *env*, and *nef* genes of HIV-1. These vectors were used to obtain amphotropic retrovirus vector producer lines that efficiently transduced each of the three HIV regulatory genes. The producer cell lines described represent a significant advance over those previously reported in that they have significantly higher titers, and only

one HIV gene is transferred in each case.<sup>35,36</sup> A retrovirus vector similar to pLTRESN containing the HIV *tat*, *rev*, and *env* genes in a single construct was described as capable of transducing all three genes.<sup>36</sup> Under our experimental conditions, and with our vector, only the *tat* gene is efficiently transferred. In the case of the vector containing the *rev* and *env* genes, pLTRESN, it was surprising to note that this construct was able to efficiently transduce only the *rev* gene and that all of the clones analyzed had deleted most of the *env* gene. Because this construct expresses functional Rev, it might have been expected that at least some of the clones isolated would contain both *rev* and *env* genes. It is unclear at this point why we did not obtain producer lines expressing both Env and Rev. However, it is possible that under our experimental conditions Rev might not be fully functional in the mouse PE501 cell line as described by other investigators in rodent cell lines.<sup>37,38</sup>

All cells infected with LtatSN were able to trans-activate the HIV LTR, demonstrating that the transduced *tat* gene was fully functional. Sequences in human chromosome 12 have been described as required for trans-activation of the HIV LTR in



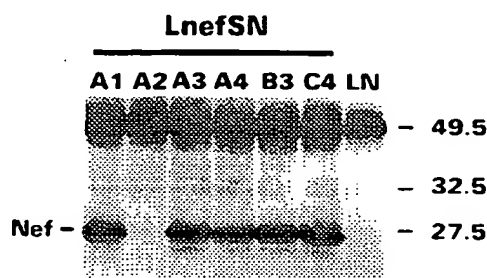
**FIG. 3.** HIV LTR trans-activation in cells transduced with LtatSN. (A) Functional Tat expression in human monocytic/macrophage (U937), B (AA2), and T (HPBALL, SupT1) cell lines transduced with LtatSN (*tat*) or a control vector (*con*). (B) HIV LTR trans-activation in human HeLa and mouse NIH 3T3 TK<sup>-</sup> cell lines (*con*), cells transduced with a control vector (*neo*), with LtatSN (*tat*), or with LrevSN (*rev*). The presence of functional Tat was determined by measuring trans-activation of an LTR-CAT construct as described in Methods. The lower spot corresponds to the [<sup>14</sup>C]chloramphenicol. The top spots correspond to the acetylated forms of [<sup>14</sup>C]chloramphenicol.

**TABLE 1.** COMPLEMENTATION OF *rev*<sup>-</sup> MUTANT OF HIV BY LrevSN<sup>a</sup>

Cell line	HIV p24 production (pg/ml)	
	Control cells	LrevSN-transduced cells
CEM	50 <sup>b</sup>	1500
Jurkat	5	3045
Molt4	20	1335
SupT1	40	3540
AA2	190	3925
HeLa	156	2106

<sup>a</sup> Rev function was assayed by measuring p24 produced by a *rev*<sup>-</sup> HIV-1 provirus introduced into the different cell lines by electroporation or transfection as described.<sup>33</sup>

<sup>b</sup> p24 (pg/ml) was measured with a commercial ELISA kit (see Materials and Methods).



**FIG. 4.** Nef expression in cells infected with LnefSN. Extracts from NIH 3T3 TK<sup>-</sup> cell clones transduced with LnefSN or a control vector (LN) were immunoprecipitated with anti-Nef rabbit antiserum and separated on an SDS-PAGE gel as described in Materials and Methods. Samples were then blotted onto nitrocellulose and probed with Nef antiserum. The blot was developed with an alkaline phosphatase-labeled goat anti-rabbit (Fc-specific) antibody. The position of the Nef protein in the gel is indicated. Migration of prestained molecular weight markers (kDa) is indicated on the right. The dark band that migrates above the 49.5-kDa marker is the Fc portion of the IgG used in the immunoprecipitation prior to Western blot.

CHO cells.<sup>39</sup> Under our experimental conditions mouse NIH 3T3 TK<sup>-</sup> cells transduced with LtatSN efficiently trans-activated the HIV LTR in the absence of human DNA sequences. These results indicate that NIH 3T3 TK<sup>-</sup> cells contain all factors required for *tat* function. Cells transduced with LrevSN expressed functional Rev as determined by its ability to complement an HIV *rev*<sup>-</sup> mutant. Of all the cell lines tested, AA2 and HeLa had the highest level of background p24 produced by the HIV *rev*<sup>-</sup> mutant. The molecular basis of this leakage is at present not understood but it might indicate the presence of a cellular factor(s) that partially complement the function of Rev. Transduction of *nef* was efficiently done by LnefSN, using standard methodology.

The ability of the retrovirus vectors described here to efficiently transfer the regulatory genes of HIV will be helpful in studying their effects on cell function as well as gene expression in representative cell populations. Because the number of vector copies integrated into the genome is low when compared to transfected populations, more homogeneous levels of expression are usually obtained, resulting in more meaningful observations. Retrovirus vectors have been used to express foreign genes *in vivo* via bone marrow transplantation or by direct injection into liver in mice.<sup>25,40</sup> Therefore the vectors described here should also be useful in the development of small animal models for the study of the role of Tat, Rev, and Nef in HIV pathogenesis and disease progression. Cell lines that constitutively express Tat and Rev will be useful to produce stocks of infectious viruses lacking either functional Tat or Rev by trans-complementation.<sup>41</sup> The use of cell lines that contain a single integrated copy of *tat* or *rev* will minimize the chance of recombinations that could produce replication-competent virus. This in turn will be useful in addressing their role in HIV latency. The availability of an efficient retrovirus vector for *nef* transfer has already proved to be useful in addressing the effect of Nef on CD4 surface levels.<sup>30,42,43</sup> More recently a similar vector has also been useful in assessing the effect of SIV Nef on human

CD4 as well.<sup>44</sup> In the future, LnefSN should be helpful in addressing the mechanism of CD4 downmodulation by Nef.

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